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APPLICATION NUMBER: *60/557,612*
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THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US60/557,612*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ *Additional inventors are being named on th____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

METHODS AND APPARATUS FOR DETERMINING MITOCHONDRIAN CONTROL REGION MUTATIONS ASSOCIATED WITH ALZHEIMER'S DISEASE

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 30 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☒ Other (specify) (Inc. Exhibit A &B);Postcard

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

FILING FEE AMOUNT ($)

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number 50-0878 — $80.00

☐ Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: Nat'l Inst. Health, USA, No. AG13154 and NS21328 and Ellison Medical Foundation, No. Senior Investigator Award

Respectfully submitted,

SIGNATURE

Date 03/29/04

REGISTRATION NO. *(if appropriate)* 32,460

Docket Number: UCIVN-061N

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Attorney Docket No. UCIVN-061N

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Wallace, et al.

Serial No.: To Be Determined

Filed: Herewith, March 29, 2004

Title: Methods and Apparatus for Determining Mitochondrial Control Region Mutations Associated with Alzheimer's Disease

Transmittal of Provisional Application for Patent
37 CFR 1.53 (b) (2)

Express Mail Mailing Label No. EV220346063US

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed, for filing in the United States Patent Office under 37 CFR 1.53 (b)(2), please find the following documents:

1. Provisional Patent Application consisting of 30 total pages (including Exhibit A and B), entitled "Methods and Apparatus for Determining Mitochondrial Control Region Mutations Associated with Alzheimer's Disease"
2. A completed Provisional Application Cover Sheet consisting of 1 page;
3. Check No. 3376 in the amount of $80.00; and
4. A Return Postcard

The inventors of the invention(s) disclosed in this Provisional Patent Application are:

Douglas C. Wallace, Ph.D.

and

Pinar E. Coskun, M.D.

All further correspondence should be mailed to applicant's undersigned counsel at the address shown here below.

Respectfully submitted,

STOUT, UXA, BUYAN & MULLINS, LLP

Date: March 29, 2004

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CERTIFICATE OF MAILING

I hereby certify that this transmittal letter and the accompanying Provisional Patent Application entitled "Methods and Apparatus for Determining Mitochondrial Control Region Mutations Associated with Alzheimer's Disease" are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on March 29, 2004 and is addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Date: March 29, 2004

By: Francine Sanders, Assistant

PROVISIONAL APPLICATION FOR UNITED STATES PATENT

by

Douglas C. Wallace

and

Pinar E. Coskun

assignors to

The Regents of The University of California

for

METHODS AND APPARATUS FOR DETERMINING MITOCHONDRIAL CONTROL REGION MUTATIONS ASSOCIATED WITH ALZHEIMER'S DISEASE

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DOCKET NO. UCIVN-061N

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METHODS AND APPARATUS FOR DETERMINING MITOCHONDRIAL CONTROL REGION MUTATIONS ASSOCIATED WITH ALZHEIMER'S DISEASE

THE INVENTION

Alzheimer Disease (AD) is the most common form of progressive dementia observed in the elderly. It is associated with the accumulation of β-amyloid (Aβ) plaques and neuritic tangles in the brain. However, the cause of late-onset AD is unknown. Also, to date, the molecular cause of late-onset AD has been unknown and, thus, no reliable molecular diagnostic test for late-onset Ad has been heretofre known.

Applicant has determined that late-onset AD is associated with the age-related accumulation of somatic mutations in the regulatory elements of the mtDNA control region (CR) that result in a 50% reduction in the light (L)-strand RNA transcripts and a 50% reduction in the mtDNA/nuclear DNA (nDNA) ratio in the cortex of AD brains. Such mutations result in chronic deficiency in mitochondrial oxidative phosphorylation (OXPHOS) and an associated increase in mitochondrial reactive oxygen species (ROS) and, thus, lead to the premature death of the cortical neurons through mitochondrial permeability transition pore (mtPTP)- induced apoptosis. Therefore, the accumulation of somatic mtDNA CR mutations is likely to be the cause of late-onset AD. Hence, the present invention provides reliable molecular diagnostic test for the presence of or pre-disposition to AD.

The mtDNA CR is a 1000 nucleotide pair (np), non-coding, region of the mtDNA that contains the promoters for the initiation of heavy (H) and L-strand transcription (P_H & P_L) , the associated mitochondrial transcription factor (mtTFA) binding sites, the three conserved sequence blocks (CSB) I-III, and the origins of H-strand replication (O_H). Hence, the CR is the primary site for the regulation of mtDNA transcription and replication.

Specific mtDNA CR mutations have been found to accumulate with age in particular tissues. A T to G transversion at np 414 (T414G) was found to

accumulate with age in human skin fibroblasts (Michikawa et al, 1999, *Science* **286**:774-779) and an A189G and a T408A mutation were observed to accumulate in skeletal muscle (Wang et al, 2001, *PNAS* **98**:4022-4027). However, the T414G mutation could not be detected in normal brain using our sensitive protein nucleic acid (PNA)-clamping polymerase chain reaction (PCR) technique (Murdock et al, 2002, *NAR* **28**:4350-4355).

However, when we tested for the T414G mutation by PNA-clamping PCR in AD brain frontal cortex, we found that 65% of the AD brains were positive for this mutation while none of the normal control brains had the mutation. To investigate this phenomenon further, we cloned and sequenced multiple CR clones from the brains of AD patients and age-matched controls. This revealed that the AD brains had a 63% overall increase in CR mutations, and these mutations were preferentially located in sequence motifs in the CR that were known to be involved in regulating mtDNA transcription and regulation. For example, we found seven mutations each in the CSBI and in the P_H & P_L mtTFA elements in AD brains but none in the control brains. Moreover, the age distribution of the AD CR mutations was distinctive, being 65% higher than controls in the ages 59-69, 14% higher in ages 70-79 brains, and 130% higher in ages 80 and older AD brains.

Beyond the overall increase in mtDNA CR somatic mutations in AD brains, we also discovered two CR mutations that were unique to the brains of AD patients, the T414C and T477C mutations. In addition, mutations at T146C, T152C, A189G, and T195C were more common in AD brains than controls. Finally, the T146C, T195C and T477C mutations increased to very high levels in the AD brains, in certain cases coming to represent 70-80% of all of the mtDNAs in the patient's brain. Moreover, these mutations were often found together in AD brains, but not in control brains. Finally, these specific mtDNA CR mutations were found at very high frequencies primarily in patients in the age range of 70 to 83 years old, the same range that had the reduced frequency of more random CR mutations. This implies that there are two classes of AD. In one case, a few CR mutations arise early in

development, become widely disseminated throughout the brain, and then clonally amplified in each cell giving rise to an earlier-onset dementia associated with a high frequency of a few mutations in the brain. In the other case, multiple mutations accumulate, but later in development so that each individual mutation is confined to a fewer number of cells. When these mutations are clonally amplified within their respective cells, each mutation can only come to represent a few percent of the total mtDNA CR mutations in the brain.

In either case, when the percentage of the mutant mtDNAs reaches a high enough level within a cell, it inhibits mtDNA transcription and/or replication. This leads to reduced L-strand transcription, inhibition of mtDNA replication, respiratory deficiency, premature neuronal death and dementia. Hence, these data indicate that somatic mtDNA CR region mutations are the cause of late-onset AD. Therefore, testing for these mutations becomes and excellent tool to confirm the diagnosis of AD or to predict those individuals who are at risk for the disease though currently pre-symptomatically.

Further aspects and details of the present invention, along with supporting experimental data and results, are found in the documents appended hereto as Exhibits A and B, the entirities of which are expressly incorporated herein and for a part of this provisional patent application.

Disclosure Statements in Claim Format:

The invention disclosed herein includes, but is not necessarily limited to that recited in the following claims:

1. A method for determining the existence of or potential for development of Alzheimer's disease (AD) or another neurological disorder associated with the development of beta amyloid plaques in a human or veterinary patient, said method comprising the step of:

A) determining the presence of mtDNA CR mutations.

2. A method according to Claim 1 above, wherein Step A comprises making a quantitative determination of mtDNS CR mutations.

3. A method according to Claim 2 further comprising the step of:

B) comparing the results of the quantitative determination made in Step A with control values representative of patients who do not have and/or do not acquire AD, to determine whether the patient has significantly more mtDNS CR mutations than control.

4. A method according to Claim 2 further comprising the step of:

B) comparing the results of the quantitative determination made in Step A with quantitative mtDNS CR values for patients who have and/or acquire AD, to determine whether the patient has or is at risk to develop late onset AD.

5. A method according to any of Claims 1-4 wherein Step A comprises testing for at least one mutation selected from the group consisting of:

i. the T4141G mutation;
ii. the T414C mutation;
iii. the T477C mutation;
iv. the T146C mutation;
v. the T152C mutation;
vi. the A189G mutation; and
vii. the T195C mutation.

6. A method according to any of Claims 1-5 wherein Step A is carried out at least in part by a technique selected from the group consisting of:

i. PNA-clamping PCR;
ii. oligonucleotide hybridization;
iii. primer extension; and
iv. restriction digestion

7. A method according to any of Claims 1-6 wherein the determination of Step A is made in a specimen of tissue, cells or body fluid selected from the group consisting of:

i. brain tissue;
ii. brain tissue from the frontal cortex;
iii. nervous tissue;
iv. nerve cells
v. blood
vi. blood cells;
vii. urine;
viii. urinary tract cells;
ix. skin;
x. skin cells;
xi. epithelium;
xii. epithelial cells; and
xiii. fibroblasts.

8. A test kit comprising reagents and/or materials useable to perform a method according to any of Claims 1-7.